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Recombinant Ig-Like Transcript 3-Fc Modulates T Cell Responses via Induction of Th Anergy and Differentiation of CD8+ T Suppressor Cells

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The Ig-like transcript (ILT)3 is crucial to the tolerogenic activity acquired by dendritic cells exposed to allospecific T suppressor (Ts) cells. We have explored the immunomodulatory property of the extracellular region of ILT3 using a cytoplasmic deletion mutant of ILT3 (ILT3Δ), expressed as membrane-bound ILT3 on KG1 cells, and a rILT3-Fc fusion protein. We found that both membrane-bound and soluble ILT3 inhibited T cell proliferation in primary and secondary MLC inducing anergy in CD4+ Th cells and suppressing the differentiation of IFN-γ-producing CD8+ CTL. Furthermore, membrane-bound and soluble ILT3 induced the differentiation of CD8+ FOXP3+ Ts cells in primary 7-day MLC. The suppressive activity of these CD8+ Ts cells is alloantigen specific and mediated by their capacity to induce the up-regulation of ILT3 and down-regulation of costimulatory molecules such as CD86 in APC from the stimulator used for priming, but not on control HLA-mismatched APC. Our finding that ILT3-Fc has potent immunosuppressive activity in vitro and that it acts on T cells only upon activation suggests the possibility that this agent may be of use for specific suppression of the immune response in autoimmunity or transplantation.

Increasing evidence demonstrates the immunosuppressive potential of CD4+ regulatory T cells (Treg) suggesting that they may be of therapeutic value (reviewed in Refs. 1–3). Naturally arising Treg express constitutively CD25, the IL-2Rα chain, and high levels of FOXP3, the X-chromosome-encoded forkhead transcription factor (1–3). Although Treg do not proliferate in vitro or produce IL-2 after TCR cross-linking, they are capable of inhibiting proliferative responses and cytokine production by effector T cells through a still unknown mechanism (2). This inhibitory capacity, however, is not Ag-specific or MHC-restricted and, thus, in the clinical setting of transplantation or autoimmune diseases, Tregs may cause nonspecific immunosuppression, a drawback of currently used iatrogenic therapy (2–4). Alloantigen-specific T suppressors (Ts) can be generated, however, both in vitro and in vivo by chronic antigenic stimulation (3, 5–8). In previous studies, we demonstrated that multiple in vitro stimulation of human T cells with allogeneic APC results in the differentiation of CD8+CD28− T cells, which are MHC class I-restricted and suppress Ag-specific CD4+ Th cell responses, inhibiting their capacity to produce IL-2 and preventing up-regulation of CD40L (9–10). We further showed that CD8+CD28− alloantigen-specific Ts express FOXP3 and induce the up-regulation of Ig-like transcript (ILT)3 and ILT4 on monocytes, dendritic cells (DC), and endothelial cells (EC), rendering them tolerogenic (11–12). Tolerogenic ILT3highILT4high DC induce anergy in alloreactive CD4+CD45R0−CD25+ T cells converting these cells into Tregs which, in turn, continue the cascade of suppression by tolerizing other DC (7).

ILT3 (also known as CD85K, LIR5) and ILT4 are members of the Ig superfamily, which are selectively expressed by professional APC such as monocytes, macrophages, and DC (13, 15), and nonprofessional APC, such as EC (12). Similar to other members of the ILT family (ILT2 and ILT5), ILT3 and ILT4 have extracellular Ig-like domains, responsible for ligand binding at the cell surface, and a long cytoplasmic tail containing ITIM, which recruit inhibitory phosphatases and transduce a negative signal into the cell (13–16).

Treatment of human DC and EC with IL-10 and/or IFN-α also induces the up-regulation of ILT3 and ILT4 and renders these cells capable of inhibiting the proliferation of allogeneic T cells (7, 11, 12, 17). The crucial role of ILT3 and ILT4 in the induction of T cell unresponsiveness was documented by overexpressing these receptors in the myelomonocytic cell line KG1. ILT3- or ILT4-transfected KG1 cells induced anergy in unprimed or KG1-primed allogeneic T cells whereas mAbs to ILT3 and ILT4 blocked their inhibitory effect on T cell proliferation (11).

The ligand of ILT3 is unknown and no ortholog of this receptor has been identified in rodents. ILT4 binds to HLA class I molecules (14, 17–19) and its murine homolog was shown to be the paired Ig-like receptor (PIR)-B (20, 21). Using an experimental model of chronic antigenic stimulation of recipient rats with UVB-irradiated blood from their donor, we demonstrated that CD8+FOXP3+ Ts cells mediate tolerance to allogeneic heart transplants, inducing PIR-B in donor DC and EC and rendering the graft resistant to rejection (8).
Based on the finding that these Ig-like receptors mediate the induction of tolerance, we have explored the effect of membrane and soluble ILT3 (ILT3-Fc) on alloactivated human T cells. We now report that membrane-bound and soluble ILT3 inhibit the generation of T cells with cytotoxic or effector function and induce the differentiation of Ts cells.

Materials and Methods

Construction and expression of ILT3 and the ILT3 Δmutant

The generation of the KG1.ILT3 cell line, which is transfected with full-length ILT3, and of KG1.MIG, transfected with empty vector, has been previously described (11). Deletion of the ITIM-containing cytoplasmic region of ILT3 was accomplished by PCR amplification using the following primers: 5′ primer CCATGATATACGAGCGACCATGATTCCCA and 3′ primer AGTGACCGCCGCTTTTCCCTGGACGTCAG. The pDN4.ILT3 plasmid containing a full-length cDNA of ILT3 was used as template. PCR conditions were as follows: 5 min 94°C; 30 cycles (30 s 94°C, 1 min 68°C, 1 min 72°C); 7 min 72°C. The PCR product was purified using a PCR purification kit (Qiagen) and subcloned into the EcoRV and NotI sites of the expression vector pcDNA4/T0/myc-His in frame with a c-myc-His epitope (Invitrogen Life Technologies). The resulting ILT3 deletion mutant, ILT3 Δmutant (M1-N328), encodes a protein which contains the putative leader peptide, the extracellular and transmembrane domains, and a stretch of 48 aa of the cytoplasmic domain of ILT3 followed by a C-terminal myc-His tag. The ILT3 Δinert was subcloned into the BglII site of retroviral vector MIG (MSCV-IRE-GFP) and the resulting construct was confirmed by sequencing. ILT3 was overexpressed in KG1 cells by retroviral transduction (11). Transfectants were sorted for GFP expression by flow cytometry and cultured in complete medium. The stable ILT3Δ-transfected cell line is referred to hereafter as KG1.ILT3 Δmutant.

Construction of a soluble form of ILT3

The extracellular domain of ILT3 fused to the Fc region of human IgG1 was integrated into the mammalian expression vector pcDNA3 (Invitrogen Life Technologies). The human IgG1 H chain C region, containing the hinge region and the CH2 and CH3 sequences, was isolated with H chain-specific primers by PCR amplification of cDNA from the RNA of human spleen. H chain-specific primers: (5′ primer TCCTGCAAAAACCTCA CACATGCC and 3′ primer TTGGCGGCCGCCGCACTTAT) were designed so that the resulting amplified fragment was flanked by a 5′ NotI and a 3′ Nol restriction sites. The extracellular 237-aa sequence of mature ILT3, preceded by the ILT3 signal sequence, was isolated by PCR amplification of pcDNA4-ILT3. ILT3-specific primers (5′ primer GAGTCGACAGAGCGACCATGATCCCA and 3′ primer GTCAGCCACAGTCGACAG) were designed so that the amplified fragment was flanked by a 5′ KpnI and a 3′ SalI restriction sites. The two amplified fragments were cloned in the pGEM-T vector (Promega), and the correct inserts were verified by sequencing. The KpnI/SalI extracellular domain of ILT3 was then fused to the SalI/Nol Fc region of human IgG1 by sequential cloning in the pBluescript KS vector (Strategene). To abolish binding to the FcRs, a mutation was introduced in the N-linked glycosylation site of the human Fc (N79S). Lastly, the KpnI/NolI sequence encoding the ILT3-Fc chimeric protein was cloned into the pcDNA3 (Invitrogen Life Technologies) mammalian expression vector.

Expression and purification of ILT3-Fc fusion protein

Expression vector pcDNA3 ILT3-Fc was linearized using BglII for transfection into CHO-S cells (Invitrogen Life Technologies). A stable transfected CHO-S cell line, expressing ILT3-Fc, was generated by transfecting 3 × 10⁶ cells with 5 μg of linear plasmid by nucleofection (Amaxa). Transfected cells were selected in serum-free medium (CHO-S-SFM II) containing 1500 μg/ml geneticin (Invitrogen Life Technologies) and screened for expression of ILT3-Fc by RT-PCR and immunoblotting. Homogenous cell populations were obtained by limiting dilution and clones with high expression of ILT3-Fc were selected. ILT3-Fc fusion protein was purified from the supernatant of the selected clones using a recombinant protein A-Sepharose fast flow column (Pierce Biotechnology), followed by dialysis against PBS and 0.22-μm filter sterilization. Purified protein was stored at −20°C before use.

Immunoblot analysis

Cells were washed, lysed, and proteins were extracted. Protein preparations were brought to equal concentrations, separated on SDS-PAGE gels and then transferred to nitrocellulose and probed with specific mAb: anti-myc (clone 9E10, Sigma-Aldrich), anti-human-Fc (Southern Biotechnology Associates), anti-FOX3 mAb (clone hFOX3, eBioscience), and anti-phosphotyrosine rabbit polyclonal Ab (Santa Cruz Biotechnology). Finally, the membranes were treated with HRP-conjugated secondary Abs and films were developed by the ECL method (Amersham Biosciences). To investigate the interaction of SHP-1 with different ILT3 constructs, cell extracts were immunoprecipitated using anti-SHP-1 antibody (Santa Cruz Biotechnology) and protein G followed by Western blot analysis with anti-myc Ab, as described above. Cells were pretreated with pervanadin (PV; 100 μM) for 30 min at 37°C where indicated. For cross-linking experiments, mAb anti-HLA-DR (BD Biosciences), anti-ILT3, and F(ab')₂ goat anti-mouse IgG (Sigma-Aldrich) were used.

T cell isolation and culture

PBMC from healthy blood donors were separated from buffy coats by density gradient centrifugation. CD3⁺ T cells were obtained by selective depletion of CD14, CD19, CD36, CD16, CD36, CD123, and glycoporphin A-positive cells using CD3 isolation kits (Miltenyi Biotec) according to the manufacturer's instructions. CD25⁺ T cells were depleted from all CD3⁺ T cell suspensions used in functional assays. CD4⁺ or CD8⁺ T cells were magnetically sorted from CD3⁺ T cells to a purity of 95–98% using CD4 or CD8 isolation kits (Miltenyi Biotec). All cell cultures were performed in complete medium (RPMI 1640 supplemented with 10% FCS, 2 mM l-glutamine, and 50 μg/ml gentamicin from Mediatech). Proliferation assays

Primary MLC were performed using responding T cells (5 × 10⁵ cells/well) and allogeneic-stimulating cells (2.5 × 10⁵ cells/well) irradiated with 3000 rad. CD2-depleted APC or the myelomonocytic cell lines KG1 (American Type Culture Collection), KG1.MIG, KG1.ILT3 (11), or KG1.ILT3 Δmut were used as stimulating cells. Cultures were harvested on day 6. For secondary MLC, responding T cells from primary MLC were collected on day 7, washed and restimulated for 3 days with the APC used for priming or with CD3⁺, as indicated. [³H]Thymidine was added to the cultures 18 h before harvesting and incorporation was determined by scintillation spectrometry using a LKB 1250 Betaplate counter (PerkinElmer). Mean cpm of triplicate cultures and the SD from the mean were calculated. In some experiments, Ts cells were added to responding cells at various ratios. Recombinant human IL-2 (10 U/ml; R&D Systems) or anti-ILT3 mAb (5 μg/ml) (clone ZM 3.8, a gift from Dr. M. Colonna, Washington University School of Medicine, St. Louis, MO) were added to the proliferation assays as indicated. The fusion protein ILT3-Fc was used at various concentrations as indicated. Normal human IgM (Sigma-Aldrich) was used at the same concentrations in parallel control cultures.

Cytolysis assay

CD3⁺CD25⁺ T cells were cultured with irradiated KG1.MIG cells in the presence or absence of ILT3-Fc (50 μg/ml) or with KG1.ILT3 Δmut cells. After 7 days of incubation, CD8⁺ T cells were isolated, washed, and cultured for an additional 6 h with KG1 cells at a ratio of 10:1 (E:T). For flow cytometry studies, the cells were labeled with anti-CD8 mAb (allophycocyanin-conjugated) from BD Biosciences for gating out CD8⁺ T cells, and with Annexin VFITC and propidium iodide (PI; BD Biosciences). Early (annexin V-positive) and late (annexin V and PI double-positive) apoptotic KG1 cells were isolated by flow cytometry. As a control, CD8⁺ T effector cells and KG1 target cells were cultured separately and mixed at the same E:T ratio before staining. For ⁵¹Cr release assays, KG1 target cells were labeled with ⁵¹Cr (0.1 μCi/ml) at 37°C for 1 h, washed, counted, and plated at 10⁵well in 0.1 ml of medium. CD8⁺ T cells tested as effectors were added at an E:T ratio of 10:1 in 0.1 ml of medium. For spontaneous and maximum release, 0.1 ml of medium or 0.2% Triton X-100 was added to each well. The plates were briefly spun and placed into a 37°C incubator. After 5 h, the plates were spun again and 0.1 ml of supernatant from each well was transferred to a glass tube and counted in a gamma counter (Packard Instrument). Specific lysis was determined using the formula: (100 × (experimental release − spontaneous release)/(maximum release − spontaneous release)).

ELISPOT assay for the production of IFN-γ, IL-2, and IL-10

ELISPOT analysis of IFN-γ, IL-2, and IL-10 was performed using cytokine-specific kits (BD Biosciences). Briefly, ELISPOT plates were coated overnight at 4°C with capture Ab at the recommended concentration. CD3⁺ T cells were isolated from CD3⁺CD25⁺ T cells which had been primed for 7 days with KG1 in the presence or absence of ILT3-Fc, KG1.ILT3, or KG1.ILT3 Δmut. CD8⁺ T cells (2 × 10⁵ cells/well) and irradiated KG1 cells (2 × 10⁵ cells/well) were plated in triplicate reactions.
Plates were incubated at 37°C for 24 h. Cells were aspirated and plates were washed twice with deionized water. Wells were soaked for 3–5 min at each wash step to lyse the remaining cells. Plates were washed again with PBS containing 0.05% Tween 20 and coated with detection Ab at room temperature for 2 h. Plates were then washed and streptavidin-HRP at the dilution recommended by the manufacturer was added to each well. After 1 h of incubation at room temperature, plates were washed and developed by adding 3-amino-9-ethyl-carbasole substrate. Developed plates were dried overnight and analyzed using ImmunoSpot software (Cellular Technology).

**Induction of CD8⁺ Ts cells by use of membrane-bound ILT3 or ILT3-Fc protein**

CD3⁺CD25⁻ T cells (1 × 10⁶/ml) were cultured with irradiated allogeneic CD2-depleted PBMCs (0.5 × 10⁶/ml) in the presence or absence of ILT3-Fc protein or control human IgG (50 μg/ml) or with irradiated KG1, KG1.ILT3, or KG1.ILT3Δ (0.5 × 10⁶ cells/well). CD8⁺ T cells were isolated from these cultures after 7 days and tested for inhibitory activity. For suppression assays, various numbers of alloactivated CD8⁺ T cells were added to primary MLC containing autologous CD3⁺DC²5⁻ T cells (5 × 10⁴ cells/well) and the allogeneic APC used for Ts priming (2.5 × 10⁶ cells/well). Proliferation was measured as described above.

**Generation of monocyte-derived DC**

Monocytes were obtained from PBMCs using a Monocyte Negative Selection kit (Dynal Biotech). Immature DC were generated by culturing monocytes for 7 days in 6-well plates at a concentration of 2 × 10⁶ cells/well with GM-CSF and IL-4 (R&D Systems). On day 7, immature DC were collected and washed then cocultured with Ts.

**Diffusion chamber experiments**

CD4⁺CD25⁻ T cells (2.5 × 10⁶ cells/well) and irradiated allogeneic APC (5 × 10⁶ cells/well) were cocultured in the bottom compartment of a Transwell system (Nalge Nunc International). CD8⁺ Ts isolated from primary MLC containing autologous CD3⁺DC²5⁻ T cells (5 × 10⁴ cells/well) and the allogeneic APC used for Ts priming (2.5 × 10⁶ cells/well). Proliferation was measured as described above.

**FACS analysis**

Flow cytometry studies were performed on a BD Biosciences FACSCalibur instrument using five-parameter acquisition (forward scatter, side scatter, and three fluorescence channels) (BD Biosciences). The following mAbs were used: CD4-FITC, CD8-FITC, CD86-FITC, CD11c-PE, CD25-PE, CD3-CyChrome (all from BD Biosciences), and ILT3-PC5 (Coulter). For each cell surface marker, a corresponding isotype-matched Ab conjugated with the same fluorescent dye was used as a negative control. Cells were incubated with saturating concentrations of the indicated mAbs for 20 min at 4°C in PBS containing 5% FCS and 0.01% NaN₃, washed twice and analyzed by FACS using CellQuest software.

ILT3-Fc was labeled with FITC dye using the FluoReporter FITC Protein Labeling kit (Invitrogen Life Technologies). The end product, ILT3-Fc-FITC, had a concentration of 0.4 mg/ml and a degree of labeling of 3:1 molecules of dye per protein. Normal mouse IgG (Caltag Laboratories), used as a negative control for ILT3-Fc-FITC staining, was dialyzed and labeled with FITC by the same method. Cells were stained by adding 10 μl of ILT3-Fc-FITC or IgG-FITC to 100 μl of cell suspension (10⁵ cells). After 30 min of incubation at 4°C, cells were washed and analyzed.

**Molecular typing of HLA class I and II**

HLA genotypes of healthy blood donors were determined by PCR with sequence-specific primers using commercially available kits (One Lambda).

**Results**

Membrane-bound ILT3b induces CD4⁺ Th cell anergy and inhibits the generation of CD8⁺ cytotoxic cells

The cytoplasmic region of ILT3 contains ITIM that recruit inhibitory phosphatases, which can negatively regulate cell activation (15). The extracellular portion consists of two Ig-like domains, which presumably enclose the ILT3 ligand-binding sites involved in the interaction of APC with T lymphocytes. We engineered a construct (ILT3Δ) that includes the extracellular and transmembrane regions but lacks the ITIM-containing cytoplasmic domain of the ILT3 receptor and is, thus, unable to deliver intracellular signals upon ligand binding. This construct was overexpressed in KG1 cells and the resulting cell line, KG1.ILT3Δ, was used to explore the activity of membrane-bound ILT3 (Fig. 1A)

KG1.ILT3 (11) and KG1.ILT3Δ expressed similar amounts of ILT3 protein on the cell surface as shown by flow cytometry analysis using mAb to ILT3 (Fig. 1B). Western blot analysis using anti-myc Ab, which binds to the COOH-terminal myc tag of the recombinant
proteins, demonstrated that the molecular masses of the ILT3 and ILT3delta protein products were 50 and 38 kDa, respectively (Fig. 1C).

The cytoplasmic region of ILT3 contains ITIM, which associate with SHP-1 protein tyrosine phosphatase mediating negative signaling. This association is increased upon ILT3 cross-linking with specific Abs or PV treatment (15). Immunoprecipitation experiments using the anti-SHP-1 Abs, followed by Western blot analysis using anti-myc Abs, showed constitutive interaction of the untruncated ILT3 molecule with SHP-1. This interaction was increased by treating KG1.ILT3 with PV. As expected, no interaction of SHP-1 with ILT3delta was observed (Fig. 1C, middle and right panels). Cross-linking of HLA-DR and ILT3 on KG1.ILT3 cells inhibited tyrosine phosphorylation, as also shown by others (15), yet had no effect on tyrosine phosphorylation in KG1.ILT3delta cells, consistent with the lack of SHP-1 recruitment (Fig. 1D).

Comparison of the ability of KG1, KG1.MIG, KG1.ILT3, and KG1.ILT3delta to elicit T cell proliferation in primary and secondary MLC showed that KG1 and KG1.MIG but not KG1.ILT3 or KG1.ILT3delta elicited proliferation of unprimed (Fig. 2A) or KG1-primed T cells (Fig. 2B). The allostimulatory activity of KG1.MIG, transfected with the empty vector, was consistently equal to that of KG1. In six repeat experiments, addition of ILT3 mAbs or rIL-2 to the blastogenesis assays restored T cell proliferation in response to KG1.ILT3 or KG1.ILT3delta. This indicates that membrane ILT3 protein is sufficient for inducing an inhibitory signal in activated T cells and that deletion of the cytoplasmic region of ILT3 does not abrogate its T cell-anergizing activity.

To study the effect of ILT3 on the differentiation of CTLs, we primed CD3+CD25+ T cells with KG1.MIG, KG1.ILT3 or KG1.ILT3delta. On day 7, CD8+ T cells from these cultures were isolated and tested for their ability to kill KG1 cells. CD8+ T cells from cultures primed with KG1.ILT3 or KG1.ILT3delta showed virtually no cytotoxic activity while CD8+ T cells primed with KG1.MIG killed KG1 cells as determined both by annexin V/PI staining (Fig. 2, C and D) and 51Cr-release assays (Fig. 2E). Analogous results were obtained using T cells from five different donors in repeat experiments. Hence, similar to full-length ILT3, ILT3delta inhibits the differentiation of CD8+ CTL.

**FIGURE 2.** T cell responses to KG1, KG1.MIG, KG1.ILT3, and KG1.ILT3delta. Proliferative responses of: A, Unprimed CD3+ T cells; B, KG1-primed CD3+ T cells cultured with or without rIL-2 or anti-ILT3. C, Killing of KG1 target cells by CD8+ T cells primed as indicated; Annexin V staining. D, Percent apoptotic cells (early plus late apoptosis) from C at a 10:1 E:T ratio. E, Percent target cell lysis in 51Cr release assay at a 10:1 E:T ratio.
ILT3-Fc protein induces Th cell anergy and inhibits the generation of CTLs

Based on the finding that the extracellular domain of ILT3 inhibits T cell function even upon deletion of the cytoplasmic region, we explored the possibility of using ILT3-Fc as an immunomodulatory agent. For this, we engineered a soluble form of ILT3, which was expressed in CHO-S cells as an Fc-fusion protein (Fig. 3A). Western blot analysis of ILT3-Fc using anti-human Fc Ab, showed that its molecular masses were 50 and 90 kDa, under reducing and nonreducing SDS-PAGE conditions, respectively, indicating the dimeric nature of ILT3-Fc (Fig. 3B).

We investigated the effect of ILT3-Fc on proliferative responses of human CD3⁺CD25⁺ T cells following primary or secondary stimulation with allogeneic APC. Addition of ILT3-Fc, at a concentration of 5 and 50 μg/ml, inhibited day 6 blastogenesis in primary MLC by 60 ± 8% and 90 ± 5%, respectively, in five independent experiments. No inhibition was observed at concentration of 0.5 μg/ml (Fig. 3C). Similarly, when ILT3-Fc was added to secondary MLC, at the time of restimulation, there was 80 ± 10% inhibition of the 3-day memory response at a concentration of 50 μg/ml (Fig. 3D). Purified human Ig, used as a specificity control in two parallel experiments, had a slight inhibitory effect on primary (15 ± 5%) and secondary MLC (17 ± 7%) only at the highest concentration tested (50 μg/ml). To further examine the specificity of the inhibitory effect displayed by ILT3-Fc, we added anti-ILT3 mAb (5 μg/ml) to cultures in which CD3⁺CD25⁺ responding cells were primed to allogeneic APC in the presence of ILT3-Fc (5 μg/ml). The proliferative response was inhibited (60%) by ILT3-Fc, yet restored when anti-ILT3 mAb was added at the initiation of the MLR. mAb to ILT3 did not enhance T cell allo-reactivity in cultures without ILT3-Fc (Fig. 3E). This indicates that the inhibitory effect displayed by ILT3-Fc is specific. To determine whether ILT3-Fc has any toxic effects, T cell viability in cultures with and without ILT3 was analyzed by annexin V/PI staining. No decrease in T cell viability after incubation with ILT3-Fc in primary or secondary MLC was observed, indicating that ILT3-Fc is not toxic.

Study of the capacity of ILT3-Fc to inhibit the differentiation of CTLs showed that CD8⁺ T cells primed in 7-day cultures with KG1.MIG cells, in the presence of ILT3-Fc (50 μg/ml), did not kill KG1 cells in annexin V/PI or ⁵¹Cr release CTL assays (Fig. 2, C and D). In contrast CD8⁺ T cells primed to KG1 in the presence or absence of human IgG displayed CTL function (Fig. 2E). Hence, similar to membrane-bound ILT3, soluble ILT3 inhibits the differentiation of CTL.

Membrane-bound and soluble ILT3 induce the generation of CD8⁺ FOXP3⁺ Ts

We investigated the possibility that CD8⁺ T cells allostimulated in the presence of membrane or soluble ILT3, not only lose CTL activity but also acquire suppressor function. To determine whether ILT3-Fc induces the in vitro differentiation of CD8⁺ Ts cells, we primed CD3⁺CD25⁺ T cells isolated from fresh peripheral blood with allogeneic APC, in the presence of ILT3-Fc or control human IgG (50 μg/ml). Increasing numbers of CD8⁺ T cells, magnetically sorted from these cultures after 7 days, were added to MLC containing unprimed CD4⁺CD25⁺ T cells from the same responder and APC from the original stimulator. CD8⁺ T cells primed in the presence of ILT3-Fc induced dose-dependent inhibition of T cell proliferation reaching >90% at a 1:1 ratio of

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**FIGURE 3.** Structural and functional analysis of rILT3-Fc. **A,** The extracellular domain of ILT3 fused to the Fc region of human IgG1 was cloned into the mammalian expression vector pcDNA3. **B,** Molecular masses of ILT3-Fc as determined by SDS-PAGE under reducing and nonreducing conditions. Supernatant of nontransfected (10 μg) (lane 1) and ILT3-Fc transfected (1, 5, 10 μg) CHO-S cells was used. **C,** Proliferative responses of unprimed and (D) primed CD3⁺ T cells to allogeneic APC in the presence of different concentrations of ILT3-Fc or human IgG. **E,** Effect of mAb to ILT3 on ILT3-Fc-induced suppression of T cell proliferation.

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primed CD8+ Ts to responding CD4+ Th cells (Fig. 4A). CD8+ T cells primed in control cultures containing purified human IgG induced at most 20% inhibition. In parallel cultures, we studied the capacity of KG1.ILT3 to induce the generation of CD8+ Ts. CD3+CD25+ T cells were primed for 7 days either with KG1 or KG1.ILT3 cells. At the end of the incubation time, CD8+ T cells were isolated and tested for their capacity to inhibit the response to KG1 of unprimed, autologous CD4+ T cells. CD8+ T cells primed to KG1.ILT3 induced dose-dependent inhibition of T cell responses to KG1, while CD8+ T cells primed to KG1 showed inhibitory activity (37%) only at the highest (1:1) Ts/Th cell ratio (Fig. 4B). These results were reproduced in four independent experiments which indicate that soluble ILT3 as well as membrane ILT3 induce the differentiation of CD8+ Ts in primary MLC.

To better characterize CD8+ Ts induced in the presence of ILT3, we analyzed the cytokine profile of CD8+ T cells cultured for 7 days with KG1, KG1 plus ILT3-Fc, KG1.ILT3 and KG1.ILT3 delta. ELISPOT assays showed that the frequency of IFN-γ-producing CD8+ T cells was much lower after priming in the presence of membrane or ILT3-Fc compared with cultures primed with KG1. A similar decrease in the frequency of IL-2-producing cells was observed in these populations. The frequency of IL-10-producing cells was also decreased although less dramatically. However, the fact that ILT3-Fc or membrane ILT3 did not induce an increase in the size of the IL-10-producing T cell population precludes the possibility that the suppressive effect of these CD8+ Ts cells is mediated by IL-10 (Fig. 4C).

To further explore the possibility that ILT3-induced Ts secrete inhibitory factors, coculture experiments using semipermeable membranes, for separating CD8+ Ts cells from CD4+CD25+ Th cells were performed. CD4+CD25+ T cells isolated from the fresh peripheral blood of responder A were stimulated with irradiated CD2-depleted APC of individual B in the bottom compartment. ILT3-Fc-induced CD8+ Ts from primary MLC (A primed to B) were added to the bottom compartment or stimulated with the same APC (of B) in the top compartment. Inhibition of alloreactivity occurred only when CD8+ Ts, CD4+ Th and APC were in close contact, but not when Ts cells and Th cells were separated by a membrane, indicating that cell-to-cell interaction is required for the suppressive effect induced by CD8+ Ts cells to occur (Fig. 4D).

 Because FOXP3 is a characteristic marker for CD4+ Treg cells (1–3, 5) and CD8+ Treg cells (12, 22), we analyzed its expression in CD8+ T cells primed for 7 days to allogeneic APC in the presence or absence of ILT3-Fc (50 μg/ml) and in CD8+ T cells primed for 7 days with KG1 or KG1.ILT3. Western blot analysis of CD8+ T cells with suppressor activity using mAb to FOXP3 showed that both ILT3-Fc and membrane ILT3 induced high expression of FOXP3 (Fig. 4E). These data indicate that ILT3-Fc and membrane ILT3 induce the differentiation of CD8+ Ts with potent inhibitory activity.

**FIGURE 4.** Study of CD8+ Ts cells primed in the presence of membrane or soluble ILT3. A, CD8+ T cells stimulated in 7-day cultures with allogeneic CD2-depleted APC in the presence of ILT3-Fc or control human IgG were tested for their ability to suppress primary MLC containing autologous responding T cells and APC from the original stimulator. Various numbers of CD8+ Ts were added to a constant number of responding and stimulating cells. B, CD8+ T cells primed for 7 days to KG1.ILT3 were tested for their ability to suppress primary MLC containing autologous T cells and KG1 stimulating cells. Various numbers of CD8+ Ts were tested for suppressive activity. C, ELISPOT analysis of IFN-γ, IL-2, and IL-10 production. CD8+ T cells primed in 7-day MLC with KG1, KG1 + ILT3-Fc, KG1.ILT3, or KG1.ILT3 delta were restimulated for 24 h with KG1. Number of positive spots per well was determined. D, Diffusion chamber experiments. ILT3-induced CD8+ Ts were tested for their effect on the proliferative response of autologous CD4+ Th to allogeneic APC. Ts and Th were stimulated with allogeneic APC either mixed together or separated by a semipermeable membrane. E, Western blot analysis of FOXP3 expression in CD8+ T cells primed with CD2-depleted APC (lane 1), CD2-depleted APC plus ILT3-Fc (lane 2), KG1 (lane 3), and KG1.ILT3 (lane 4).
Mechanism of action of ILT3-Fc-induced CD8$^+$ Ts

CD8$^+$ Ts generated by multiple in vitro stimulation with allogeneic APC were previously shown to act directly on APC, inducing the down-regulation of costimulatory molecules and the up-regulation of ILT3 and ILT4 (11). To determine whether CD8$^+$ Ts generated by allostimulation in the presence of ILT3-Fc have a similar effect on APC, we tested their ability to modulate CD86 and ILT3 expression on DC from the donor used for priming and on control DC from an individual sharing no HLA class I Ags with the original stimulator.

CD8$^+$ T cells isolated from the culture containing ILT3-Fc were able to dramatically up-regulate the expression of ILT3 on DC from the specific stimulator but not on control DC. This allelotype-specific up-regulation of the inhibitory receptor ILT3 occurred in conjunction with the down-regulation of CD86 (Fig. 5A).

To determine whether the generation of CD8$^+$ Ts is due to ILT3 binding to a putative ligand on CD8$^+$ T cells or whether it is secondary to ILT3 binding to CD4$^+$ T cells, we labeled ILT3-Fc protein with FITC and used it for staining T cells before and after stimulation in primary MLC. Unprimed T cells from five different individuals showed ILT3-Fc binding on 2–5% of the cells. Three days after allostimulation, up to 26% of CD4$^+$ T cells were stained by ILT3-Fc-FITC (Fig. 5B). CD8$^+$ T cells from the same cultures showed only 2–3% ILT3-Fc-binding before and 1–7 days after allostimulation. These results suggest that ILT3-Fc binds primarily to activated CD4$^+$ Th cells rendering them anergic and, thus, unable to help CD8 T cells to differentiate into cytotoxic cells.

The direct effect of ILT3-Fc on CD4$^+$ T cells was demonstrated in experiments in which ILT3-Fc was added to cultures containing sorted CD4$^+$CD25$^+$ T cells and CD2-depleted APC from an allogeneic donor. ILT3-Fc induced 86 ± 9% inhibition at 50 μg/ml in five independent experiments while purified human IgG had no suppressive effect (Fig. 5C).

Discussion

Effective priming of CD4$^+$ T lymphocytes requires cell-to-cell interaction between APC and T cells. This interaction allows bidirectional costimulatory signals which activate the APC and T cells with cognate-specific TCR. TCR triggering results in up-regulation of CD40L expression on activated CD4$^+$ T cells. CD40L interacts with CD40 on APC inducing them to up-regulate costimulatory molecules which, upon interaction with their counterreceptor trigger CD4 Th cell proliferation and differentiation of effector function (reviewed in Ref. 2). There are numerous potentially relevant ligands whose expression is up-regulated on APC upon activation, including B7.1/B7.2 (CD80/CD86), ICOS, ICAM-1 (CD54), 4-1 BBL, OX40L, and CD70 among others. The B7.1/B7.2:CD28/CTLA-4 costimulatory pathway has received considerable attention as a regulatory point in T cell reactivity, representing attractive targets for new therapeutic strategies aimed at treatment of tumors, autoimmune diseases, and graft rejection (23–26).

The differentiation of CD8$^+$ CTL was shown to be contingent upon recognition of MHC class I/peptide complexes on APC, and notably DC, that have been “licensed” or conditioned by CD4$^+$ Th cells via the CD40-signaling pathway. There is ample evidence that T cell “help” is required for CD8 T cell activation, maturation, proliferation, and maintenance of CTL function (27–31).

However, APC can also serve a tolerogenic function (reviewed in Ref. 23). The role of ILT in induction of tolerance has been established by the finding that allospecific CD8$^+$CD28$^+$ Ts up-regulate ILT3 and ILT4 expression on DC, rendering them tolerogenic (11). Such tolerogenic DC anergize alloreactive CD4$^+$ T cells converting them into regulatory cells, which, in turn continue the cascade of suppression by tolerizing other DC (7). Similarly, alloantigen-specific CD8$^+$CD28$^+$ FOXP3$^+$ Ts have been shown to induce ILT3$^+$ILT4$^+$ tolerogenic endothelial cells, inhibiting alloreactivity (12).
with 1.25(OH)$_2$D$_4$ and its analogs can also induce DC which express inhibitory ILT and display tolerogenic properties in vitro and/or in vivo (7, 32–35).

In the present study, we have explored the immunomodulatory property of the extracellular region of ILT3 using either ILT3-Fc or a cytoplasmic deletion mutant of ILT3, expressed as membrane-bound ILT3 on KG1 cells. We found that both membrane and soluble ILT3 inhibited CD3$^+$ T cell proliferation in primary and secondary MLC inducing anergy in the responding population and suppressing the differentiation of IFN-γ-producing CD8$^+$ CTL.

The inhibitory activity of ILT3 is most likely caused by its interaction with the ILT3 ligand expressed by CD4$^+$ T cells. This view is supported by the finding that ILT3-Fc: 1) binds to a relatively large percentage of CD4$^+$ T cells but not of CD8$^+$ T cells stimulated for 3 days with allogeneic APC and 2) inhibits the response of CD4$^+$ T cells in primary MLC. It therefore appears that inhibition of CD8$^+$ CTL differentiation in cultures containing ILT3-Fc is due to lack of T cell help, rather than to a direct effect on CD8$^+$ T cells.

Importantly, however, CD8$^+$ T cells sorted from CD3$^+$-responding cells that have been allostimulated for 7 days in the presence of ILT3-Fc or ILT3β acquired suppressor function. When transferred to primary MLC, these CD8$^+$ Ts inhibited T cell proliferation in a dose-related, contact-dependent, and IL-10 independent manner. The suppressive activity of these CD8$^+$ Ts seems to be mediated by their capacity to induce the up-regulation of ILT3 and down-regulation of costimulatory molecules (such as CD86) in the APC used for priming. Because no such changes are induced in control APC with a different HLA phenotype, it results that the suppressive activity of CD8$^+$ Ts is Ag-specific and cytokine-independent. It, therefore, appears that the inhibitory receptor ILT3 can signal both intracellularly, negatively regulating activation of APC, and extracellularly via a ligand expressed by activated T cells.

Similar to CD8$^+$ Ts generated by chronic antigenic stimulation, CD8$^+$ Ts induced in the presence of ILT3 express FOXP3. Al -

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Similar to CD8$^+$ Ts generated by chronic antigenic stimulation, CD8$^+$ Ts induced in the presence of ILT3 express FOXP3. Although it is still unknown how FOXP3 induces suppression it is worthwhile noticing that it is expressed at high levels by Ts and/or in vivo (7, 32–35).

Based on our present findings, we postulate that ILT3-Fc may prove to be an important agent for induction of Ag-specific tolerance. By rendering activated CD4$^+$ T cells anergic, triggering the differentiation of CD8$^+$ Ts which induce tolerogenic DC (with high membrane levels of ILT3 and ILT4), ILT3-Fc may increase the arsenal of modern immunomodulatory agents.

Disclosures

The authors have no financial conflict of interest.

References


ILT3-Fc INDUCES CD8⁺FOXP3⁺ Ts CELL DIFFERENTIATION


